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In vitro digested milk proteins: Evaluation of angiotensin-1-converting enzyme inhibitory and antioxidant activities, peptidomic profile, and mucin gene expression in HT29-MTX cells

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Giromini, C., Lovegrove, J. A., Givens, D. I., Rebucci, R., Pinotti, L., Maffioli, E., Tedeschi, G., Sundaram, T. S. and Baldi, A. (2019) In vitro digested milk proteins: Evaluation of angiotensin-1-converting enzyme inhibitory and antioxidant activities, peptidomic profile, and mucin gene expression in HT29-MTX cells. Journal of Dairy Science, 102 (12). pp. 10760-10771. ISSN 1525-3198 doi: https://doi.org/10.3168/jds.2019-16833 Available at https://centaur.reading.ac.uk/86437/

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To link to this article DOI: http://dx.doi.org/10.3168/jds.2019-16833

Publisher: Elsevier

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Interpretative Summary

In vitro digested milk proteins: evaluation of Angiotensin-1-Converting Enzyme Inhibitory and antioxidant activities, peptidomic profile and mucin gene expression in HT29-MTX cells

by Giromini et al. We studied the bioactive effect of *in vitro* digested whey protein isolate (WPI), casein proteins (CP) and soy proteins (CTR) in term of Angiotensin-1-Converting Enzyme Inhibitory activity (ACE1-I), antioxidant (AOX) and intestinal HT29-MTX-E12 cell metabolic activity and mucus-production. Our results indicate that milk proteins possess higher AOX and ACE1-I activity after *in vitro* digestion. Peptidomic analysis reveal that WPI and CP generated potentially bioactive peptides mainly associated to ACE1-I bioactivity. We also reported that specific concentrations of WPI, CP and CTR are able to promote HT29-MTX-E12 cells metabolic activity and, in the case of CP, the MUC5AC and MUC2 gene expression, suggesting that the consumption of milk proteins can have a positive effect on intestinal defenses.

Running head: Milk protein digestion: bioactivity and peptidomics

In vitro digested milk proteins: evaluation of Angiotensin-1-Converting Enzyme Inhibitory and antioxidant activities, peptidomic profile and mucin gene expression in HT29-MTX cells

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ABSTRACT

Over the past decades, a number of studies investigated the health-promoting functions of milk peptides. However, to date many hurdles still exist regarding the widespread utilization of milk-derived bioactive peptides as they may be degraded during gastrointestinal digestion. Thus, the aim our study was to *in vitro* digest intact Whey Proteins Isolate (WPI) and Casein Proteins (CP), mimicking *in vivo* digestion, to investigate their bioactive effects and to identify the potential peptides involved. Whey protein isolate and CP were digested using a pepsin/pancreatin protocol and ultra-filtered (3kDa cut-off membrane). A permeate (<3kDa) and a retentate (>3kDa) were obtained. Soya protein was included as a control (CTR). Angiotensin-1-Converting Enzyme Inhibitory (ACE1-I) and Antioxidant activity (AOX) were assessed and compared with those observed in undigested proteins and CTR. Furthermore, the permeate was characterized by LC-nano ESI MS/MS using a shotgun-peptidomic approach while retentate was further digested with trypsin and analysed by mass spectrometry with a shotgun-proteomic approach to identify potentially bioactive peptides. Further, the effect of WPI, CP and CTR retentate on cell metabolic activity and on mucus production (MUC5AC and MUC 2 gene expression) was assessed in intestinal goblet HT29-MTX-E12 cells. Results showed that WPI permeate induced a significant ACE1-I inhibitory effect (49.2 ± 0.64 %) compared with undigested WPI, CP permeate and retentate ($P<0.001$) and with CTR permeate (10.40 ± 1.07 %; $P<0.001$). A significant increase in AOX (1.58 ± 0.04 and 1.61 ± 0.02 µmol trolox AOX equivalents /mg protein, respectively) upon digestion was found in WPI. Potentially bioactive peptides associated with ACE1-I and anti-hypertensive effects were identified in WPI permeate and CP retentate. WPI, CP and CTR retentate, at specific concentrations, were able to stimulate ($P<0.05$) metabolic activity in HT29-MTX-E12 cells; MUC5AC expression was increased ($P<0.05$) by CP retentate and unaltered ($P>0.1$) by WPI retentate. MUC2 expression was significantly increased by 0.33 mg/g CP and reduced by 1.33

mg/g CP. Our results confirm that milk proteins may be rich sources of bioactive compounds with a greatest beneficial potential of CP at intestinal goblet cell level.

KEYWORDS: milk protein; *in vitro* digestion; peptidomic; angiotensin-I-converting enzyme.

INTRODUCTION

Milk proteins are considered one of the most important source of bioactive peptides. Beyond their well-known nutritional values, milk proteins exhibit an extensive range of physiological effects that promote general health and the functions of specific organs and tissues (Meisel and FitzGerald, 2003; Martínez-Maqueda et al., 2012b). The most relevant functional properties of milk proteins are obtained following gastrointestinal digestion and milk peptides release (Baldi et al., 2005; Vermeirssen et al., 2003). A recent study compared the peptidome of human jejunal effluents after ingestion of casein and whey proteins with the peptidome obtained after their *in vitro* gastrointestinal digestion. This study has allowed the identification of protein domains that are resistant to gastrointestinal digestion (Sanchón et al., 2018). Some of these peptide fragments correspond to previously described sequences that might exert their physiological function at the intestinal epithelium level or after absorption where they can elicit unique effects (Shimizu, 2004). It has been reported that peptides from the hydrolysis of milk proteins possess functional properties such as antioxidant, angiotensin-I-converting enzyme inhibitory activity, antimicrobial and cytomodulatory activities (Baldi et al., 2005; Martìnez-Maqueda et al., 2012b; Giromini et al., 2017). Petrat-Melin et al. (2015) found that beta-casein variants exhibited antioxidant and angiotensin-I-converting enzyme inhibitory activity upon digestion. Furthermore, larger peptides from milk protein digestion pool (i.e >3kDa) are unlikely to reach the bloodstream and will therefore mediate their bioactive effect locally, at gastro intestinal level (Miner-Williams et al., 2014). Beta-casomorphin 7, a casein-derived peptide, exhibited an enhanced mucin gene expression mediated by opioid receptors in intestinal goblet cells (Zoghbi

et al., 2006). To date, however, most attention has been focused on the bioactive effect of single peptides or hydrolysed proteins (Martínez-Maqueda et al., 2012a; Plaisaincié et al., 2015) with limited data on the effect of the whole pool of peptides obtained after *in vitro* digestion of milk proteins (Mukhopadhya et al., 2015; Volstatova et al., 2016), their potential mechanism of action and the candidate peptides involved. Casein and whey proteins, however, are present in milk and dairy products in their intact form and the total peptide pool produced from their digestion may have diverse effects *in vivo.* Evidences from robust *in vitro* studies simulating protein digestion, and the subsequent evaluation of the bioactive effect could offer invaluable information on the physiological mechanisms by which these proteins may elicit their biological effect *in vivo*. Consequently, we investigated the ACE1-Inhibitory (ACE1-I) and antioxidant (AOX) activity of *in vitro* digested (permeate and retentate fractions) Whey Protein Isolate (WPI) and Casein Proteins (CP) compared to undigested samples and to soya protein isolate (CTR). A peptidomic/ proteomic analysis of WPI and CP was also performed to substantiate the presence of bioactive peptides. Further, the effect of WPI, CP and CTR retentate was tested on cell metabolic activity and on mucus production (MUC5AC and MUC2 gene expression) in HT29-MTX-E12 cells as a human intestinal goblet cell model.

MATERIALS AND METHODS

Samples and reagents

The samples tested in the study were the following: Whey Protein Isolate sample (WPI) (Volac Int Ltd, Cambridge) with a protein content of 93g/100g protein; Calcium caseinate (casein protein, CP, Garret Ingredients) with a protein content of 90g/100g protein; and soya protein isolate (CTR) (MyProtein, Northwich) with a protein content of 90g/100g.

In vitro digestion

Whey protein isolate, CP and CTR in *vitro* digestion was performed according to the method of Minekus et al. (2014), and further adapted by our group (Giromini et al., 2017).

Briefly, 20 g of WPI, CP or CTR sample were mixed with 150 mL of distilled H2O and maintained on an orbital shaker at 150 rpm for 5 minutes. The digestion procedure involved three phases. For the oral phase 6.66 mg α-amylase in 2.1 mL of 1 mM CaCl2, pH 7 was added to the samples and they were incubated for 30 min at 37 ◦C on a shaker. For the gastric phase, the pH was decreased up to 2 with 6 M HCl and 0.9 g of pepsin in 8.3 mL of 0.1 M HCl was added. The samples were then incubated for 120 min at 37 ◦C on a shaker. For the small intestinal phase, the pH was increased to 7 with 6 M NaOH and 0.2 mg pancreatin and 1.2 g bile in NaHCO3 0.5 M were added to the samples before the final incubation of 180 min at 37 ◦C on a shaker was performed.

A blank sample (enzymes of the digestion alone), a positive control and negative control were included as reference samples and for stability tests in all digestions performed (n=3).

Samples preparation

At the end of the digestion, the total digesta obtained was transferred to 3kDa cut-off membrane (VIVASPIN 20 Sartorius). Each filter was previously activated with 0.1% BSA solution. Samples were centrifuged for 20 min at 3500 x g to obtain permeate (peptides and polypeptides < 3kDa) and retentate (peptide and polypeptides > 3kDa) fractions. Aliquots from permeate and retentate fractions were sampled and snap frozen in liquid nitrogen to stop enzyme activity before storing at -80 °C for further experiments.

Angiotensin-1-converting enzyme inhibitory activity

The ACE1-I of WPI, CP and CTR undigested, permeate and retentate samples was quantified using the ACE1-I assay with furanacroloyl-Phe-Glu-Glu (FAPGG) as the synthetic substrate for the ACE1-I enzyme, as described by Giromini et al. (2017). A synthetic specific ACE inhibitor captopril at the concentration of 20nM was included as control. Data are expressed as percentage of ACE1-I (% ACE1-I).

Total antioxidant capacity-ABTS assay

Antioxidant capacity (AOX) was determined in WPI, CP and CTR undigested, permeate and retentate samples using the method described by Re et al. (1999) with modifications. Trolox stock solution (2.5 mM in distilled water) was used to produce the standard curve. A solution of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid (ABTS) (7 mM) was prepared with potassium persulfate (140 mM) in distilled water and left to react in the dark for 12–16 hours to produce the ABTS•+solution. For the study of AOX capacity of WPI, CP and CTR permeate and retentate, the ABTS•+solution was diluted with phosphate buffered saline, pH 7.4, (PBS) to reach the absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. A volume of 20µL of sample or Trolox standard was mixed with 2mL of ABTS•+ working solution and incubated in dark for 6 min at room temperature before measuring absorbance at 734 nm on the spectrophotometer. Appropriate solvent blanks were included in each assay. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of samples and of Trolox for the standard reference data. AOX results are expressed as µmol Trolox equivalent (TE)/ mg protein.

Proteomic and peptidomic profile

Permeate and retentate of WPI and CP were analysed by LC-nano ESI tandem mass spectroscopy using a shotgun-label free approach to identify peptides and proteins. The permeate was analysed without any digestion before MS/MS to identify endogenous peptides presents in the samples, the so called peptidomic strategy (Aletti et al. 2016) (Fig. 1) while the retentate was further digested using sequence-grade trypsin with a protein:protease ratio 20:1 (Iametti et al., 2001) with a proteomic strategy (Fig. 1). The digestion with trypsin is a normal procedure in the proteomic MS/MS analysis in order to identify the proteins based on the sequence of the corresponding tryptic peptides. Trypsin generates experimentally observable peptides that can be used to uniquely identify a protein.

157 Mass analysis was carried out using an LTQ OrbitrapVelos (Thermo Fisher Scientific, Bremen,
158 Germany) as described in Maffioli et al. (2017) for the peptidomic strategy and in Coccetti et
159 al., (2008) for the proteomic analysis.

160 **FIGURE 1**

161 ***Peptidomic and proteomic data analysis***

162 MS spectra were searched against the mammalian NCBI sequence database (release
163 24.01.2017) (casein and whey samples) by the Sequest search engine contained in the Proteome
164 Discoverer 1.4.0 software (Thermo Fisher Scientific Inc., USA). The following parameters
165 were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, Met oxidation, N-terminal
166 acetylation and Gln /Asn deamidation as variable modifications. Carbamidomethylation of Cys
167 as fixed modification and trypsin (2 misses) as protease were parameters further added in the
168 proteomic analysis. Only peptides with False Discovery Rate 1% (against decoy) and Xcorr 1.5
169 were included for positive identification (Dell'Orco et al. 2016). Two replicates were carried
170 out for each sample in the MS analysis. All peptides were searched in SATPdb (Singh et al.
171 2016) and BIOPEP-UWM database (Minkiewicz et al 2008), two databases of structurally
172 annotated therapeutic peptides, to find potentially bioactive peptides. In order to consider
173 possible further proteolysis, the search was performed keeping a minimum sequence length of
174 6 amino acids and applying a "IF" nested function to a matrix which compared the sequence
175 of each peptide found with the ones of the database (Microsoft Excel 2016 (version 15.27).
176 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
177 Consortium via the PRIDE partner repository (Vizcaino et al 2016) with the dataset identifier
178 PXD012588 for the permeate and PXD012625 for the retentate.

179 ***Cell culture experiments***

180 In our study, the permeate and retentate fractions obtained after IVD reproduced the pool of
181 peptides generated during the physiological digestion in humans (Giromini et al., 2019). Small
182 peptides (<3KDa, permeate fraction) could be absorbed by epithelial cells along the small

intestine and enter the blood circulation, whereas polypeptides (>3KDa, retentate fraction) can reach the colonic environment. Thus, in a second set of experiments we tested the effect of WPI, CP and CTR retentate fractions on intestinal HT29-MTX-E12 cell metabolic activity and mucus production modulation.

Metabolic activity

The HT29-MTX-E12 cells were kindly donated by Dr. Stig Purup, Aarhus University, Research Centre Foulum, DK. This human colorectal adenocarcinoma cell line, which is a clone of HT29 cells, is able to differentiate into a mucus-producing goblet-like cell line. Cell maintenance was performed in 25 cm^2 flasks using 15 mL Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) and supplemented with 10% of FCS, 1% 1 M HEPES, 1% (v/v) penicillin/streptomycin, 2% Glutamax and 1% MEM NEAA. The cells were cultivated at 37°C in a humidified incubator with 5% CO_2. All experiments were performed using HT29-MTX-E12 cells within six cell passages (passages 40 to 45) to ensure reproducibility.

Undifferentiated HT29-MTX-E12 cells were plated at a density of $1.5x10^4$ cells/well in 96-well plates and cultured for 24 hours. Whey protein isolate, CP and CTR retentate were diluted in 0.05% FCS DMEM (Giromini et al., 2015) and added to the cell culture at different concentrations (from 42.62 to 0.16 mg/ml). For the control wells, 0.05% FCS DMEM was added.

The retentate stock solutions of WPI, CP and CTR contained between 86.23 and 85.24 mg protein/ml. Stock solutions have been normalised to 85.24 mg/ml, which corresponds to the lowest protein concentration observed among WPI, CP and CTR. Considering the high cytotoxicity of stock solutions and the absence of DMEM medium to regulate the osmotic pressure, we have tested diluted concentrations of retentate from 42.62 up to 0.16mg/ml.

After 3 and 24 hours of treatment, the metabolic activity of HT29-MTX-E12 cells was evaluated by MTT test. MTT assay measures the production of the chromophore formazan from 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide (MTT). Formazan is produced

209 in viable cells by the mitochondrial enzyme succinate dehydrogenase. Three replicates per
210 treatment were included and the experiments were repeated three times (n=3).
211 Specifically, the cell metabolic activity percentage induced by the protein retentate
212 wascalculated as follows:
213 % cell metabolic activity = (mean optical density of treated cells / mean optical density of
214 control cells) x 100.
215 ***Mucus production- real-time quantitative PCR (qRT-PCR)***
216 The HT29-MTX-E12 cells were seeded at a density of $5x10^5$ cells/well in 12-well plates and
217 maintained for up to 21 days in complete medium (Martinez-Maqueda et al., 2012a). Martinez-
218 Maqueda et al. (2012a) reported that, after 21 days of culture, HT29-MTX-E12 cells express a
219 stable amount of mucus. At day 21, the cells were washed with PBS, and the medium was
220 replaced with 0.05% FCS DMEM medium for 24 h (cell starvation). The treatment medium
221 (0.05% FCS) with WPI, CP or CTR protein retentate at different concentrations (0.33 up to
222 1.33 mg/g) was added to the cells, which were then incubated in a controlled atmosphere (37°C
223 / 5% CO_2) for 3 hours. The concentration range was chosen based on the MTT test results.
224 Control cells (0mg/g) were not treated.
225 qRT-PCR assay was carried out with the real-time fluorescence method using a Strategene
226 Mx3000p. At the end of 3 hours incubation, total RNA was extracted from the HT29-MTX-
227 E12 cells using a nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to the
228 manufacturer's protocol. The RNA (50ng) was reverse transcribed using the iScript cDNA
229 synthesis kit (BioRad), and the resulting cDNA was used as a template for qRT-PCR. MUC5AC
230 and MUC2 genes were amplified by qRT-PCR with primer sequences that have previously been
231 published by Martínez-Maqueda et al.(2012a) and Nielsen et al.(2018) (Table 1), and
232 cyclophilin and β -actin were included as a reference genes. The primers have been checked for
233 specificity in BLAST before the Real-time PCR experiments.
234 **TABLE 1**

Each reaction tube contained 2X SYBR Green real-time PCR Master Mix, gene-specific forward and reverse primers and the cDNA (1µl). The master mix included Maxima® Hot Start Taq DNA polymerase, dNTPs in an optimized PCR buffer, and SYBR® Green I dye supplemented with ROX passive reference dye. All reactions were analyzed under the same conditions and normalized to the ROX reference dye to correct eventually fluctuations in the readings due to evaporation phenomena. The samples were tested in triplicate and non-reverse transcribed controls and no-template controls were included in the assays. The thermal profile began with 4 minutes at 95°C followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 minute. Relative quantification was performed, and the values were normalized to the internal reference gene cyclophilin. Two internal controls, cyclophilin and β-actin genes were tested as endogenous genes and cyclophilin was selected because its amplification was more efficient and had no variation across treatments. This verified the efficiency of its use as the endogenous control.

Statistical analysis

Statistical analysis were performed using GraphPad-Prism version 8 software package (GraphPad Software, San Diego, USA). Data are presented as least square means ± SEM. ACE1-I and AOX data were analysed using a two-way ANOVA to identify the significant differences between undigested, permeate and retentate in WPI, CP and CTR. The differences between means were compared using Tukey's test and considered statistically significant at $P<0.05$. For gene expression analysis, the comparative CT method was used (Livak and Schmittgen, 2001) to determine the fold changes in gene expression, which were calculated with the threshold method ($2^{-\Delta\Delta CT}$). A two-way ANOVA was also performed on cell metabolic activity and gene expression data and means were compared using Tukey's test ($P< 0.05$).

RESULTS

In vitro digestion

WPI, CP and CTR samples were *in vitro* digested and the total digesta was filtered with a 3kDa membrane to obtained a permeate (<3kDa) and a retentate (>3kDa) fraction. The WPI, CP and CTR permeate fractions were further tested for ACE1-I and AOX activities. The WPI, CP and CTR retentate fractions were tested for ACE1-I, AOX and cytomodulatory effect. A peptidomic/proteomic characterization was performed on WPI and CP permeate and retentate to check the presence of bioactive peptides.

Angiotensin-1-Converting Enzyme Inhibitory activity

Whey protein isolate permeate exhibited the highest ACE1-I effect. In particular, WPI permeate induced a significant ACE1-I inhibitory effect (49.2 ± 0.64 %) compared with WPI undigested ($P<0.001$), CP permeate ($P<0.001$) and CP retentate ($P<0.001$) and with CTR permeate (10.40 ± 1.07 %; $P<0.001$). CP permeate induced a significant ACE1-I effect (23.91 ± 0.64%) compared with CTR permeate ($P<0.001$), which showed the lowest ACE1-I effect.

FIGURE 2

Total antioxidant capacity-ABTS assay

Whey protein isolate and CP permeate showed a significant increase in AOX (1.58 ± 0.04 and 1.61 ± 0.02 µmol trolox AOX equivalents /mg protein (TrAOX equ/mg P) respectively, compared with undigested WPI and CP (< 0.6 µmol TrAOX equ/mg protein; $P=0.02$), CTR permeate (1.07 ± 0.04 µmol TrAOX equ/mg protein; $P=0.04$) and WPI, CP and CTR retentate. Low AOX was observed in CTR retentate (<0.6 µmol TrAOX equ/mg protein).

Proteomic/ peptidomic analysis of digesta

Based on the results obtained with ACE1-I and AOX activities, WPI and CP permeate and retentate samples were analyzed for their peptide content. Permeate was characterized by LC-nano ESI MS/MS using a shotgun-peptidomic approach while retentate was further digested with trypsin and analysed by mass spectrometry with a shotgun-proteomic approach as shown in Fig.1. The list of the peptides identified in the retentate and permeate of WPI and CP are reported in the Supplementary Tables S1 (permeate) and S2 (retentate), respectively. All the data sets from CP and WPI were searched in SATPdb and BIOPEP-UWM databases taking into account possible further proteolysis to find potentially bioactive peptides. In accordance with the results reported above, peptides with ace inhibitory and anti-hypertensive activity were identified in the WPI permeate together with one peptide with anti-hypertensive properties in the casein retentate (TPEVDDEALE) (Table 2).

TABLE 2

Cell culture experiments

In our study, the retentate fraction contained molecules with a molecular size larger than 3kDa, these molecules can reach the colonic tissue and ideally can escape the small intestinal absorption. There is little evidence that dietary bioactive peptides other than di and tri peptides, can cross the gut wall and enter the blood circulation. Di-and tri- peptides can be absorbed by selective transport systems (such as paracellular pathway via tight junctions, passive diffusion via enterocytes, endocytosis or by carrier-mediated transport systems), larger peptides and protein particulates may cross the gut wall though only in very small quantities (Miner-Williams et al., 2014). Therefore, we selected the >3kDa retentate fraction for colon cell experiments.

The results presented here indicated that all of the protein fractions, at specific concentrations, were able to modulate the metabolic activity of the HT29-MTX-E12 cells and to exert trophic effects on the intestinal epithelia. Treatment of HT29-MTX-E12 cell monolayer with WPI, CP and CTR (soya protein isolate) retentate caused stimulation or reduction of cell metabolic activity depending on the dosage, as measured by MTT test (mitochondrial activity). As shown in Figure 3, in the lowest range of concentrations tested (0.16mg/g-1.33mg/g), the CTR retentate was effective in significantly enhancing HT29-MTX-E12 cell metabolic activity compared with untreated cells (0 mg/g). In particular after 24 hours, CTR retentate at the concentrations from 0.16 to 0.66 mg/g significantly enhanced cell metabolic activity ($P<0.01$). After 24 hours treatment, CP at the concentration of 0.33 and 0.66 mg/g increased cell metabolic activity compared with untreated cells ($P<0.05$); whereas after 3 hours of CP and WPI and after 24 h WPI treatment cell metabolic activity remained unchanged compared with untreated cells (0 mg/g). Considering the highest concentration range (2.66-21.31mg/g), we observed that, WPI, CP and CTR retentate reduced the metabolic activity of the HT29-MTX-E12 cells in a dose-dependent manner. In contrast, CTR retentate after 3 h treatment at the concentration from 2.66 up to 21.31 mg/g maintained cell metabolic activity compared with untreated cells (0mg/g).

MUC2 and MUC5AC gene expression

FIGURE 4

We cultured HT29-MTX-E12 cells for 3 h in the presence of WPI, CP or CTR retentate. Specific concentrations of CP retentate modulated the expression of mucins MUC5AC and MUC2 in HT29-MTX-E12 cells. In particular, CP retentate promoted the expression of MUC5AC mRNA at 0.33 mg/g ($P= 0.02$), 0.66 mg/g ($P=0.04$) and 1.33 mg/g ($P= 0.008$) compared with untreated cells (0 mg/g) and with WPI and CTR. MUC5AC mRNA expression

328 remained unaltered (P>0.1) by WPI and CTR retentate treatment. CP retentate at the
329 concentration of 0.33mg/g also promoted the expression of MUC2 mRNA. The MUC2 mRNA
330 gene expression by WPI and CTR retentate remained unaltered.

331 **DISCUSSION**

332 In the present study we *in vitro* digested WPI, CP and soya (CTR) proteins to assess their ACE1-
333 I and AOX activities and to study the release of the bioactive peptides.
334 We reported that permeate and, to a lesser extent, retentate of WPI showed a significant ACE1-
335 I activity compared with undigested WPI, demonstrating that the *in vitro* digestion influenced
336 the liberation of ACE1-I peptides. Furthermore, also the permeate fraction of CP showed higher
337 ACE1-I activity after digestion, confirming the results reported in our previous study where the
338 ACE1-I activity was tested on a panel of dairy and plant protein supplements (Giromini et al.,
339 2017), and in the study of Petrat-Melin et al. (2015). In the CTR sample, the ACE1-I activity
340 was significantly reduced in permeate fraction, compared with CTR undigested. For peptides
341 to be effective ACE1-inhibitors, they must resist digestion and enter the circulation before
342 reaching the target organ. Therefore, the bioactivities observed *in vitro* may not directly
343 translate into significant effects *in vivo*, due to the differential bioavailability of peptides in
344 different individuals. The heterogeneity in individuals' responsiveness to proteins consumption
345 can prevent the identification of the association between dietary intakes and health, hinder the
346 identification of health benefits for specific population groups and limit our understanding of
347 the exact role of the different bioactive compounds. Moreover, also the absorption step in the
348 gastro-intestinal environment represents a key point in dairy peptide bioactivity. A bioactive
349 peptides to exert ACE1-I effect after oral administration must remain intact and active after
350 intestinal transport and brush border peptidases contact.
351 As reported by Miner-Williams et al (2014), only a few of the great number of milk peptides
352 with proven antihypertensive activity *in vitro* have so far proven to be clinically effective *in*

353 *vivo*. However, our recent studies (Fekete et al., 2016a; 2016b and 2018) demonstrated a
354 relationship between blood pressure reduction in humans as a result of chronic and acute
355 consumption of milk proteins-based supplements and their *in vitro* ACE1-I activity. Those
356 studies demonstrated a significant decrease in blood pressure in both whey and casein protein-
357 consumers groups, compared with the control group (no protein) in a double-blind cross-over
358 randomized controlled trial. The results reported in the present study further support the
359 aforementioned outcomes.
360 In the present study *in vitro* digestion significantly enhanced AOX capacity of WPI, CP and
361 CTR as evaluated by ABTS assay. In our experimental conditions, CP permeate and to a lesser
362 extent WPI permeate showed higher AOX properties, compared with CTR. Caseins have high
363 content of antioxidant amino acids as tyrosine and tryptophan, the oxidation of which may
364 quench free radicals inducing AOX effect (Clausen et al., 2009). Clausen et al. (2009) also
365 reported that caseins are the highest radical scavengers in milk. Di Pierro et al. (2014) reported
366 that the AOX capacity values for NaCN increased from 0.06 to 0.18 μmol trolox equivalents/mg
367 protein and for β-casein from 0.51 to 1.19 μmol trolox equivalents/mg protein, after enzymatic
368 hydrolysis. Petrat-Melin and co-authors (2015) assessed the AOX of *in vitro* digested β-casein
369 variants and they reported a significant increase in AOX capacity upon digestion. The AOX
370 capacity exhibited by the permeate of WPI, CP and CTR in this study enforces the claim that
371 food proteins are a natural source of AOX compounds. The distinct AOX properties of our
372 samples may be attributed to the specificity of the peptides or amino acids released from WPI,
373 CP and CTR primary sequences.
374 Based on ACE1-I and AOX activity results, we further analysed WPI and CP permeate and
375 retentate for their bioactive peptide content. In accordance with the results reported above,
376 peptides with ACE1-I and anti-hypertensive activity were identified in the WPI permeate
377 together with one peptide with anti-hypertensive property in the CP retentate. Only a few of
378 previous studies, such as those conducted by Petrat-Melin and co-authors (2015), assessed the

379 release of bioactive peptides after digestion. A lack of information about the comparison
380 between the protein bioactivity and the effective release of peptides associated to that
381 bioactivity exists. Therefore, the present study compared the biological activities of the digested
382 proteins with the identification of bioactive peptides. All together our data confirm WPI is a
383 rich source of ACE1-I peptides with tested bioactivity, produced upon digestion. Casein protein
384 permeate showed a significant ACE1-I activity upon digestion, although no bioactive peptides
385 associated to this activity have been found in permeate. With regard to antioxidant capacity,
386 although we observed a significant increase of AOX capacity after protein digestion, we did
387 not identify peptides associated to this bioactivity. This may be due to analytical limitations
388 associated with MS peptide identification as our method allowed to identify 6 or more amino
389 acids long peptides, shorter peptides or single amino acids may have not being identified.
390 A step forward in peptidomic analysis will be also to test ACE1-I peptides from WPI and CP
391 for their absorption capacity and for their ability of preserving their bioactivity after absorption
392 and cell internalization. This will massively help in the design of nutritional strategies to prevent
393 cardiovascular disease or to standardize the industrial production of ACE1-I peptides obtained
394 by enzymatic hydrolysis.
395 In our study, the retentate fraction contained molecules with a molecular size larger than 3kDa,
396 these molecules can ideally escape the small intestinal absorption and reach the colonic tissue.
397 There is little evidence that dietary bioactive peptides other than di and tri peptides, can cross
398 the gut wall and enter the blood circulation. Di-and tri- peptides can be absorbed by selective
399 transport systems (such as paracellular pathway via tight junctions, passive diffusion via
400 enterocytes, endocytosis or by carrier-mediated transport systems), larger peptides and protein
401 particulates may cross the gut wall though only in very small quantities (Miner-Williams et al.,
402 2014). Therefore, we selected the >3kDa retentate fraction for colon cell experiments.
403 Whey protein isolate ,CP and CTR retentate at the lower range of concentrations, maintained
404 or stimulated metabolic activity of undifferentiated HT29-MTX-E12 intestinal cells; whereas

the higher range of concentrations reduced cell metabolic activity. Several food-derived peptides have been described to exert potential chemo-preventative properties against the viability of malignant cells (Meisel and FitzGerald, 2003; Fernández-Tomé et al., 2018). In this respect, the inhibitory role that we detected may be interesting from the perspective that the HT29-MTX-E12 cells have a cancerous origin.

Moreover, we observed a hormetic effect induced by CTR retentate (3 and 24 hours treatment) and, to a lesser extent, by CP retentate (24 hours treatment) in HT29-MTX-E12 cells. The hormetic response is defined as a biological phenomenon whereby a stimulatory effect results from exposure to low doses of a compounds that is otherwise inhibitory when given to a cell at higher doses (Calabrese and Baldwin, 2002; Purup and Nielsen, 2012).

In general, *in vitro* cell-based models represent an effective tool to test nutritional ingredients for food and feed evaluation (Cheli et al., 2015; Giromini et al., 2016; Fusi et al., 2018). Cell culture studies have provided increasing evidence that milk-derived bioactive peptides modulate metabolic activity, differentiation and/or apoptosis of different cell types (Hartmann and Meisel, 2007). Pecorini and co-authors (2009) demonstrated that lactoferrin could be involved in regulating the growth of both intestinal and mammary epithelial cells. Purup et al. (2007) showed that whey fractions from bovine milk stimulated intestinal cells growth. Furthermore, Meisel and FitzGerald (2003) hypothesized that milk peptides can cooperatively stimulate the viability of intestinal cells, thus, the development of the digestive tract. In a recent study, Purup and co-authors (2018) demonstrated that both casein and whey based supplements exerted proliferative effects on intestinal cells, in agreement with our study. Tonolo et al. (2018) reported the ability of milk peptides to increase cell proliferation. In general, cell proliferation is an essential mechanism for the re-establishment of the surface epithelium after injury and food proteins may have an important role in prevention and in the recovery of gut tissue integrity. However, whether the herein observed effect on cell metabolic activity is the result of individual

peptides activity or of the synergistic activity of a peptide pool produced by *in vitro* digestion is at present not known, and would need further investigations.

In our study, CP was able to significantly promote the expression of MUC5AC gene at all concentrations tested in mucus-producing HT29-MTX-E12 cells (21 days of culture). Further, we have found a relationship of the MUC2 expression effect of CP to dose: at the highest concentrations tested, CP reduced MUC2 expression while at the lowest concentration tested CP significantly improved MUC2 expression. Our results are in agreement with those of previous studies that have demonstrated the role of milk peptides in the modulation of gastrointestinal mucus production. Plaisancié et al. (2013) demonstrated that the total peptide pool from yoghurt modulated cell proliferation and the secretion of mucins in HT29-MTX-E12 cells. Martínez-Maqueda et al. (2013) reported that casein hydrolysate stimulated HT29-MTX-E12 cells and promotes the expression of MUC5AC. The same group demonstrated the mucin-secreting role of whey protein hydrolysate in HT29-MTX-E12 cells: β-lactorphin increased the synthesis of mucin proteins without eliciting differences in MUC5AC gene expression (Martínez-Maqueda 2012a). Plaisancié et al. (2015) demonstrated an increase in mucin secretion and MUC2 and MUC4 gene expression in HT29-MTX-E12. β-casomorphin-7 increases MUC5AC mRNA expression and the secretion of this mucin, as demonstrated by Zoghbi et al. (2006). α-lactorphin, a whey-derived peptide, showed enhanced expression of the MUC5AC gene in HT29-MTX-E12 cells (Martínez-Maqueda et al., 2012a).

The main limitation of our gene expression analysis is related to the lack of demonstrating mucin secretion effect, which may precede gene expression stimulation (Martinez-Maqueda et al., 2013) in the process of replenishing the intracellular mucin pool of goblet cells. This latter point requires further analysis.

Overall, the proliferative and gene expression effects we observed in this study require further investigations before the full influence of milk protein retentate can be determined at the intestinal colon cells level.

CONCLUSIONS

Our study combines the identification of bioactive peptides by both a peptidomic and a proteomic approach with *in vitro* bioactivities evaluated after simulated digestion. In summary, our data show that whey proteins have the highest angiotensin-1-converting enzyme inhibitory activity. Further, we found that whey proteins, after *in vitro* digestion, generate functional peptides preserved, in particular those related to angiotensin-1-converting enzyme inhibitory effect. This makes it possible to hypothesise that whey proteins can have similar effect *in vivo.*

Casein and soya proteins have stimulatory effects on cell metabolic activity in undifferentiated HT29-MTX-E12 cells and, in particular, we found that casein proteins may promote mucus related-gene expression in differentiated HT29-MTX-E12 cells. Furthermore, as whey, casein and soya are effective in quite low concentrations, they may be useful as functional food ingredients for the treatment of gut injury caused by inflammatory bowel diseases or diarrhoea in new-borns mammals.

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Figure legends:

Figure 1:

Peptidomic and proteomic workflow on casein (CP), whey (WPI) and soy (CTR) proteins. Retentates and permeates were analyzed by tandem mass spectroscopy using an LTQ OrbitrapVelos and searched against the mammalia NCBI sequence database (casein and whey samples) and UniProt glycine max (soy samples) by Sequest.

Figure 2:

ACE1- inhibitory effects of undigested (UND), permeate (P) and retentate (R) of WPI, CP and soy (CTR). Data are normalized on the nitrogen content of each sample and are expressed as percentage of ACE1-I. The synthetic ACE inhibitor captopril at the concentration of 20nM, included as control, inhibited 97% enzyme activity. Different superscript letters denote significant differences ($P < 0.05$). ACEi, angiotensin-converting enzyme-1.

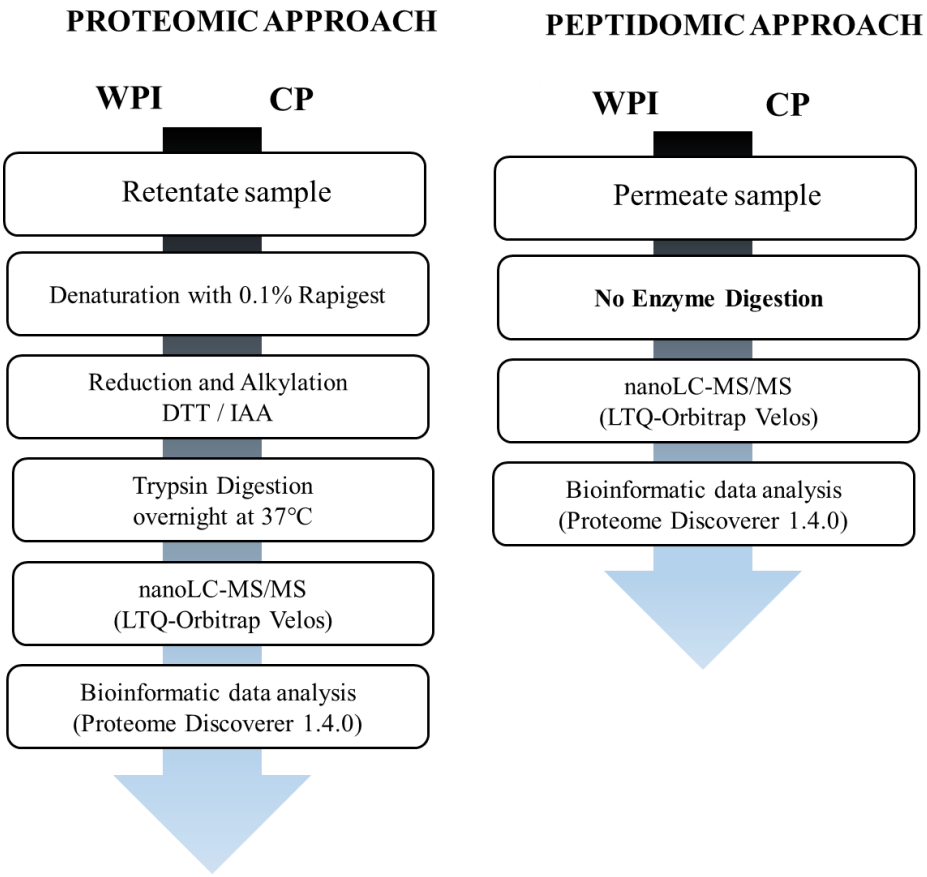
Figure 3:

Effects of WPI retentate (A), CP retentate (B) and CTR retentate (C) on HT29-MTX-E12 cell metabolic activity (expressed as cell viability) after 3 and 24 h of treatment. Data are expressed as means ± SEM. Different superscript letters denote significant differences from the metabolic activity obtained in the control cells (0 mg/ml) ($P < 0.05$).

Figure 4:

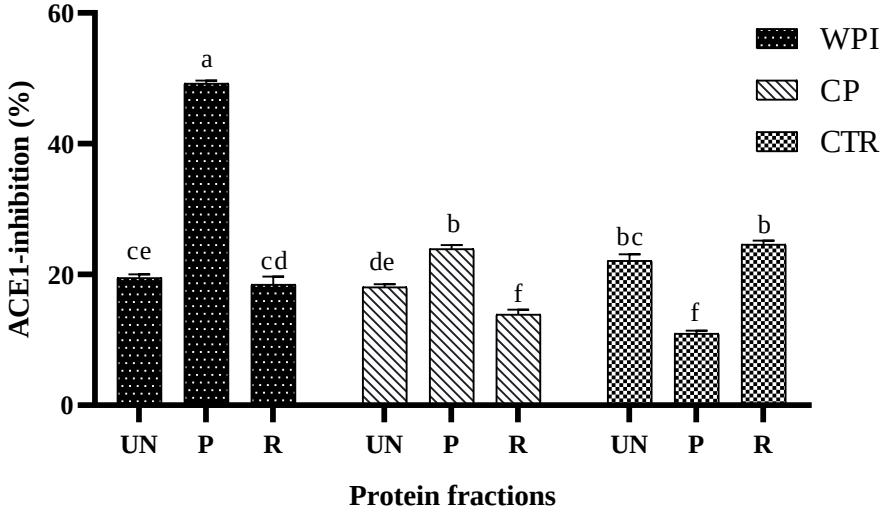
Effects of 3 hours treatment with WPI retentate, CP retentate and CTR retentate on MUC5AC (A) and MUC (B) gene expression in the HT29-MTX-E12 cells (concentrations range 0.33-1.33 mg/g) determined by qRT-PCR. The data are expressed as the relative gene expression levels relative to the controls (untreated cells, 0 mg/g). Different superscript letters denote significant differences ($P < 0.05$).

657 **Figure 1**



658

660 **Figure 2**



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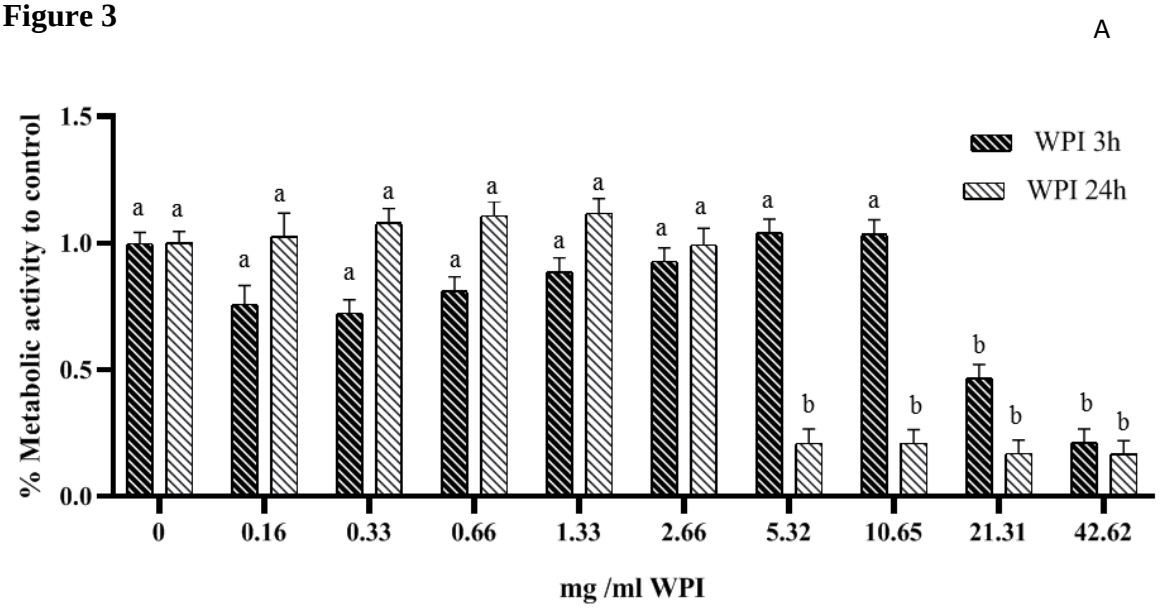
663 **Figure 3**



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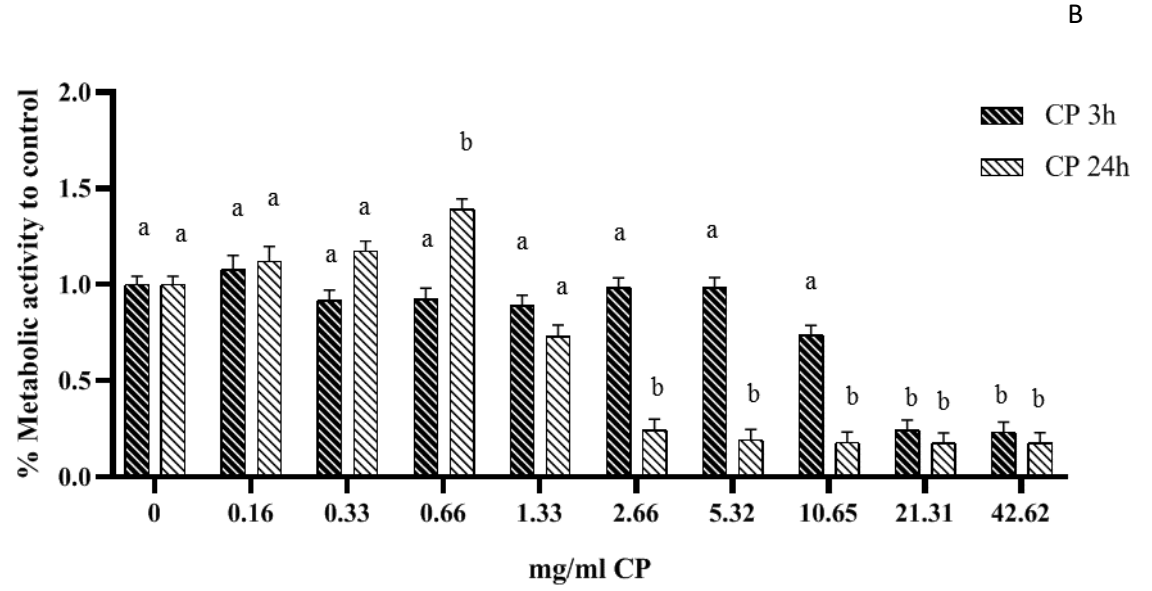

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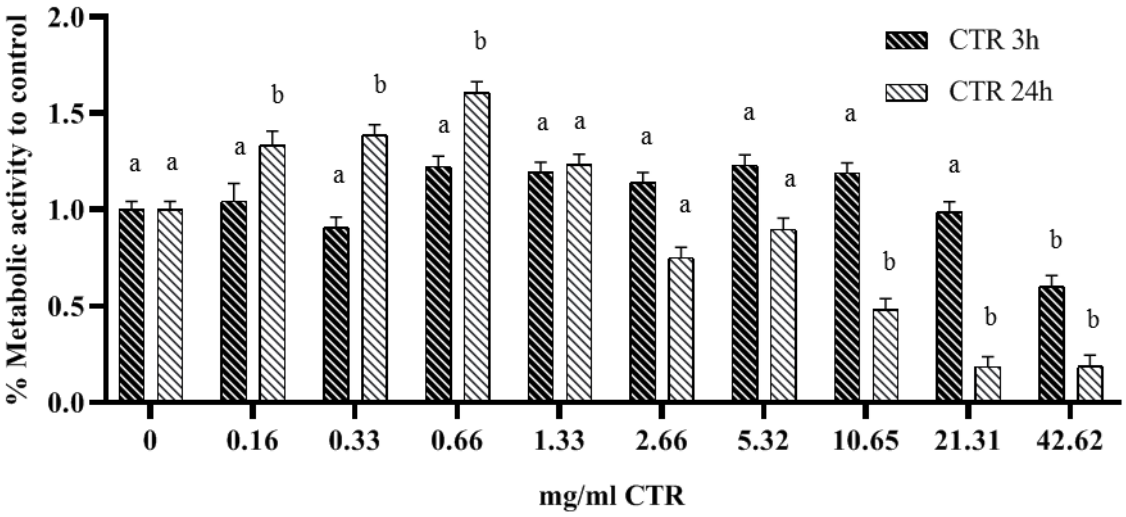
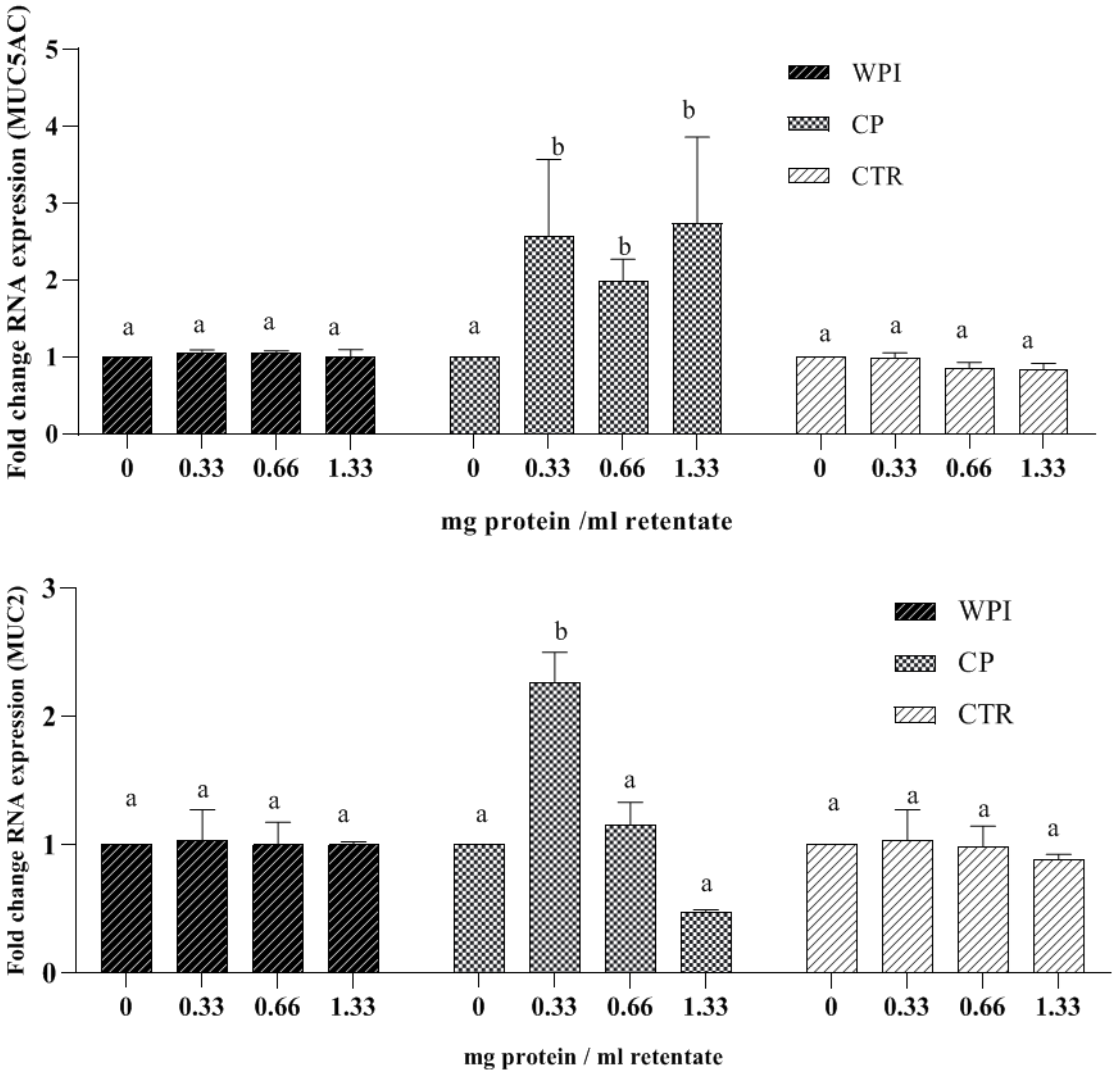
C


% Metabolic activity to control
2.0
1.5
1.0
0.5
0.0
CTR 3h
CTR 24h
a a
a b
a b
a b
a a
a a
a a
a b
a b
b b
0
0.16
0.33
0.66
1.33
2.66
5.32
10.65
21.31
42.62
mg/ml CTR

666

668 **Figure 4**



669

670

672 **Table 1**

Gene (&bp)	Primers	References
Mucin-5AC (MUC5AC) 240bp	5'- CGACCTGTGCTGTGTACCAT-3′ 3′-CCACCTCGGTGTAGCTGAA-5'	Martínez-Maqueda et al., 2012a
Mucin-2 (MUC 2)	5'- ACCCCAAGCCCTTCTACGAG-3′ 3′-GAGTGGATGCCGTTGATGGT-5'	Nielsen et al., 2018
β-actin (197bp)	5′-CTTCCTGGGCATGGAGTC-3′ 3′-GCAATGATCTTGATCTTCATTGTG-5′	Martínez-Maqueda et al., 2012a
Cyclophillin (160bp)	5′-CTTCCTGGGCATGGAGTC-3′ 3′-GCAATGATCTTGATCTTCATTGTG-5′	Martínez-Maqueda et al., 2012a

673

675 **Table 2**

	DataBase	Permeate	Retentate
Casein protein (CP)			
Anti hypertensive	TPEVDDEALE TPEVDDEALE		TPEVDDEALEK TPEVDDEALEKFDK
Whey protein isolate (WPI)			
Anti hypertensive	IDALNENK VLDTDYK LKPTPEGN VEELKP LKPTPEGN VEELKP VYVEELKPTPE	KIDALNENKVLVLDTDYK KIDALNENKVLVLDTDYK VEELKPTPEGNLE VEELKPTPEGNLE VYVEELKPTPEGNLE VYVEELKPTPEGNLE VYVEELKPTPEGNLE	
Ace inhibitory	VLDTDYK	KIDALNENKVLVLDTDYK	

676

677 **Table legends**

678 **TABLE 1.** Primer sequences used for quantitative real time polymerase chain reaction.
679 Quantification by SYBR Green was used for all genes.

680 **TABLE 2.** List of Anti-hypertensive and ACE-Inhibitory peptides found in WPI and CP
681 permeate and retentate samples.

682 **TABLE S1: List of the peptides identified in the permeate of Whey protein isolate (WPI**
683 **and casein protein (CP).**

684 Permeate was analysed by LC-nano ESI tandem mass spectroscopy using a shotgun ~~-label free~~
685 approach to identify ~~and quantify~~ peptides without any digestion before MS/MS with a
686 peptidomic approach. MS spectra were searched against the mammalia NCBI sequence
687 database (release 24.01.2017) (casein and whey samples) and UniProt glycine max (release
688 21.02.2018) (soy samples) by Sequest. Only peptides with False Discovery Rate 1% (against
689 decoy) and Xcorr ≥1.5 were included for positive identification . Two replicates were carried
690 out for each sample in the MS analysis. PSMs displays the total number of identified peptide
691 sequences (peptide spectrum matches) for the corresponding protein, including those
692 redundantly identified.

693 **TABLE S2: List of the peptides identified in the retentate of Whey protein isolate (WPI**
694 **and casein protein (CP).**

695 Retentate was analysed by LC-nano ESI tandem mass spectroscopy using a shotgun-label free
696 approach to identify ~~and quantify~~ proteins and peptides with a proteomic approach. MS spectra
697 were searched against the mammalia NCBI sequence database (release 24.01.2017) (casein and
698 whey samples) and UniProt glycine max (release 21.02.2018) (soy samples) by Sequest. Only
699 peptides with False Discovery Rate 1% (against decoy) and Xcorr ≥ 1.5 were included for
700 positive identification . Two replicates were carried out for each sample in the MS analysis.

701 PSMs displays the total number of identified peptide sequences (peptide spectrum matches) for
702 the corresponding protein, including those redundantly identified.

704 **Table S1**

PERMEATE					
WP1					
	Accession	**Description**	**Coverage**	**Unique Peptides**	
	5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	19,14	6	
		Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
		YVEELKPTPEGnLE	N12(Deamidated)	4,86	1618,77910	2
		TPEVDnEALEKFDK	N6(Deamidated)	4,68	1635,76689	1
		VRTPEVDnEALEKFDK	N8(Deamidated)	4,55	1890,93682	5
		TPEVDnEALEKFD	N6(Deamidated)	4,47	1507,67900	3
		TPEVDnEALEKFDKA	N6(Deamidated)	4,18	1706,80022	2
		VEELKPTPEGnLE	N11(Deamidated)	4,08	1455,71477	8
		VEELKPTPEGNLE		4,07	1454,73503	1
	Accession	**Description**	**Coverage**	**Unique Peptides**	
	XP_010615055.1	sodium channel protein type 4 subunit alpha (predicted)	2,07	1	

	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	GVNLFAGKFYYCI NTTtSERFDISEVN NKSECDSLmYt	T17(Phospho); M36(Oxidation); T38(Phospho)	6,03	4535,88361	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_01981 4470.1	NAC-alpha domain-containing protein 1 isoform X3 (predicted)	1,28	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sPPLQDTDLPLVqG SVsEAsPEPQSEED LTASPP	N-Term(Acetyl); Q13(Deamidated) ; S17(Phospho); S20(Phospho)	5,97	3720,59292	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_01061 0262.1	insulin receptor substrate 1 (predicted)	3,60	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	gAAELAAHSsLLG GPqGPGGMsAFTR VNLSPNRNQSAKV IRADS	N-Term(Acetyl); S10(Phospho); Q16(Deamidated) ; S22(Phospho)	5,74	4593,16811	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_01951 5287.1	keratin, type II cytoskeletal 1	8,17	1		

	isoform X2 (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	GSKSISISVAGGGR RsGFGGGYGGsGF GGGGFGGSGFGG GFGSGGFGGGFGS G	S16(Phospho); S25(Phospho)	5,70	4644,95571	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_01978 2783.1	histone-lysine N-methyltransferase EHMT2 (predicted)	11,55	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	GRILmGHATKSFP SSPSKGGACPSRA KMsMTGAGKsPPS VqSLA	M5(Oxidation); S29(Phospho); S36(Phospho); Q41(Deamidated)	5,67	4535,15249	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_01906 2163.1	helicase POLQ-like isoform X1 (predicted)	6,56	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	SVsEKFNLPRGsIQn LLTGAAsFSSCVLH FCEELEEFW	S3(Phospho); S12(Phospho); N15(Deamidated) ; S22(Phospho)	5,63	4515,94692	1

Accession	Description	Coverage	Unique Peptides		
XP_019818048.1	osteopontin (predicted)	5,04	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	RIsHELDsASSEVN	S3(Phospho); S8(Phospho)	4,63	1703,67278	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
P02670.2	Kappa-casein;	7,29	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	AIVNTVDNPEAsSE	S12(Phospho)	4,39	1525,63445	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
D3ZGB1.1	T-cell transcription factor NFAT5	1,16	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VSGNETSTTttPqVAtPG	T10(Phospho); T11(Phospho); Q13(Deamidated); T16(Phospho)	4,25	1988,70659	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019308010.1	PDZ domain-containing RING finger protein 4 isoform X1 (predicted)	2,19	1		

	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	ISEsGKLsDQEQsSSEH	S4(Phospho); S8(Phospho); S13(Phospho)	4,08	2087,73418	1

CP

	Accession	**Description**	**Coverage**	**Unique Peptides**		
	P02662.2	Alpha-S1-casein	35,51	17		
		Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
		SDIPNPIGSEnSEKTTM	N11(Deamidated)	4,40062 3322	1820,81267	4
		LHSMKEGIHAQQKEPMIGV		4,35386 1332	2133,082079	1
		SDIPNPIGSENSEKTTM		4,34833 3359	1819,83452	1
		EGIHAQQKEPMIGVNQE		4,20515 1558	1907,922533	1
		YTDAPSFSDIPNPIGSENSEK		4,19215 5838	2268,011278	1
		EGIHAQQKEPMIGVNQELA		4,09921 7415	2092,045702	4
		YVPLGTQYTDAPS		4,09641 5997	1411,665941	1
		aPSFSDIPnPIGSENSEK	N-Term(Acetyl); N9(Deamidated)	3,91413 9986	1931,870409	1
		EGIHAQQKEPMIGVNqEL	Q16(Deamidated)	3,89798 3789	2021,982225	4

	DAPSFSDIPNPIGSENSEK		3,872433901	2003,899828	2
	PSFSDIPNPIGSENSEK		3,838029146	1817,84038	1
	LHSMKEGIHAQQKEPMIGVNQE		3,784930229	2504,234911	2
	SDIPNPIGSEnSEK	N11(Deamidated)	3,743878603	1487,68059	1
	SFSDIPNPIGSENSEK		3,7101264	1720,789598	1
	DIGSESTEDQAMEDIK		3,693603516	1767,755785	1
	AMEDIKqMEAE	Q7(Deamidated)	3,629513741	1295,541063	1
	EGIHAQQKEPMIGVnQELA	N15(Deamidated)	3,586070538	2093,042528	2
	TDAPSFSDIPNPIGSENSEK		3,577449322	2104,950243	1
	SDIPNPIGSEnSEKTT	N11(Deamidated)	3,5172019	1689,766771	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019818429.1	beta-casein (predicted)	27,23	10		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	IEKFQSEEQQQTEDELQDK		5,001895905	2352,073582	1
	KIEKFQSEEQQQTEDELQDK		4,803929806	2480,175449	1

	FQSEEQQQTEDELQD		4,690680027	1853,763597	1
	FQSEEQQQTEDELQDK		4,519019127	1981,858677	2
	FQSEEQQQTEDEL		4,168885708	1610,674364	1
	FQSEEQQQTEDELQ		3,851104736	1738,735643	1
	IEKFQSEEQQQTEDEL		3,736754656	1980,895067	1
	YQEPVLGPVRGPFPIIV		3,64272213	1881,063524	1
	TEDELQDKIHPFAQTQ		3,589448452	1899,897386	1
	YPVEPFTESqSLTLTD	Q10(Deamidated)	3,553974628	1827,859667	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
5EEE_A	Chain A, Bovine Lactoglobulin Complex With Decanol	24,07	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VEELKPTPEGDLE		4,159810066	1455,715868	1
	YVEELKPTPEGDLE		4,153544426	1618,773851	2
	TPEVDDEALEKFDK		4,076670647	1635,772997	1

	VEELKPTPEGDL		4,00140 1901	1326,669481	1
	SLLDAQSAPLR		3,64488 7447	1170,645433	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	24,07	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	VEELKPTPEGnLE	N11(Deamidated)	4,15981 0066	1455,715868	1
	YVEELKPTPEGnLE	N12(Deamidated)	4,15354 4426	1618,773851	2
	TPEVDnEALEKFD K	N6(Deamidated)	4,07667 0647	1635,772997	1
	VEELKPTPEGnL	N11(Deamidated)	4,00140 1901	1326,669481	1
	SLLDAQSAPLR		3,64488 7447	1170,645433	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
P02668.1	Casoxin-C;	10	2		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	ASGEPTSTPTTEAV ESTV		4,26080 5607	1763,809496	2
	IASGEPTSTPTTEA VEST		3,57952 6186	1777,827806	1

Accession	Description	Coverage	Unique Peptides		
O88799.1	Zonadhesin;	0,8	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	gYVVHNHKCVLqIHCGCKDAQGGFVPAGKTWISRGCTQSCACV	N-Term(Acetyl); Q12(Deamidated)	5,289813519	4604,144722	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019780943.1	zinc finger protein 211-like (predicted)	5,81	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	WEEWCLLDEVqIHLYLDVmVENFALVCMLGNS	Q11(Deamidated); M19(Oxidation)	4,714330673	3828,778463	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_018872274.1	ras and Rab interactor 2 (predicted)	4,6	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sGGRPGAGPELELGTAGSPGGAPPEAAPGDCTRAPPPSS	N-Term(Acetyl)	4,624342918	3567,642233	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019822162.1	serine/threonine-protein kinase WNK2 isoform X12 (predicted)	1,75	1		

	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	qAGAGPTAASDPCGKAVqTqQPCSVRASLSADIC	Q1(Deamidated); Q18(Deamidated); Q20(Deamidated)	4,297834873	3291,473484	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_018880271.1	Golgi integral membrane protein 4 isoform X4 (predicted)	2,99	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	EENLPDENEEQKqSNqKQEN	Q13(Deamidated); Q16(Deamidated)	4,249049187	2432,032444	2
Accession	**Description**	**Coverage**	**Unique Peptides**		
NP_001034734.1	aspartate beta-hydroxylase domain-containing protein 1 isoform 2 (predicted)	25,69	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	IPPGCELVVGGEPqCWAEGHCLLVDDSF	Q14(Deamidated)	4,236132145	2971,359531	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019843937.1	pancreatic secretory granule membrane	3	1		

	major glycoprotein GP2 (predicted)				
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	DSTISVEENGVSAESR		3,794215202	1679,768846	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
P81265.1	Polymeric immunoglobulin receptor (predicted)	1,59	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	NLDTVTKEDEGW		3,734676361	1406,641771	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019818430.1	alpha-S2-casein (predicted)	5,86	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	LTEEEKNRLNFLK		3,72728014	1633,886034	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_019671843.1	zinc finger protein 462 isoform X2 (predicted)	0,89	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	dFAQDIDINPGAVYKCRHC	N-Term(Acetyl)	3,700817823	2206,984911	1

Accession	Description	Coverage	Unique Peptides		
XP_019780354.1	gem-associated protein 5 isoform X4 (predicted)	1,19	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sDqETEEEAREPELPCGV	N-Term(Acetyl); Q3(Deamidated)	3,53098 2018	2060,866991	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
XP_018895067.1	proline-rich protein 20A-like (predicted)	6,1	1		
	Peptide Sequence	Modifications	XCorr	MH+ [Da]	# PSMs
	sVEADGPAqPAqP	N-Term(Acetyl); Q9(Deamidated); Q12(Deamidated)	3,52627 7065	1310,577684	1

705

707 **Table S2**

RETENTATE

WPI

	Accession	**Description**	**Coverage**	**Unique Peptides**		
	Q01523.1	Defensin-5	12,77	1		
		Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
		ESLSGVCEISGR	C7(Carbamidomethyl)	3,107130766	1293,606737	1
	Accession	**Description**	**Coverage**	**Unique Peptides**		
	2OXS_A	Chain A, Crystal Structure Of The Trypsin Complex With Benzamidine At High Temperature (35 C)	8,97	1		
		Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
		LGEDNINVVEGNEQFISASK		3,179255962	2163,057909	1
	Accession	**Description**	**Coverage**	**Unique Peptides**		
	XP_019844211.1	39S ribosomal protein L43, mitochondrial (predicted)	8,18	1		
		Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
		EVQNPAPTQRPAQ	Q3(Deamidated); N4(Deamidated)	3,381425858	1437,700365	1

CP

	Accession	Description	Coverage	Unique Peptides		
	O02772.3	Fatty acid-binding protein 3	9,77	1		
		Peptide Sequence	Modifications	Xcorr A2	MH+ [Da]	# PSMs
		LGVEFDETTADDR		3,4	1467,64629	1
	Accession	**Description**	**Coverage**	**Unique Peptides**		
	5EEE_A	Chain A, Bovine Lactoglobulin Complex With Decanol	8,64	2		
		Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
		TPEVDDEALEK		3,684055805	1245,574877	1
		TPEVDDEALEKFDK		4,296661377	1635,76787	5
	Accession	**Description**	**Coverage**	**Unique Peptides**		
	5EED_B	Chain B, Ovine Lactoglobulin Complex With Decanol	8,64	1		
		Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
		TPEVDNEALEK	N6(Deamidated)	3,684055805	1245,574877	1
		TPEVDNEALEKFDK	N6(Deamidated)	4,296661377	1635,76787	5
	Accession	**Description**	**Coverage**	**Unique Peptides**		
	P11839.3	Beta-casein	7,21	1		

	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	FQSEEQQQTEDELQDK		4,379714012	1981,842747	1
Accession	**Description**	**Coverage**	**Unique Peptides**		
Q4GZT4.2	ATP-binding cassette sub-family G member 2	2,29	1		
	Peptide Sequence	Modifications	Xcorr	MH+ [Da]	# PSMs
	EDIGDEANETEEPSK		3,325044632	1662,693895	1

708